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A method for fixation of Plasmodium falciparum infected erythrocytes and solubilization of the erythrocyte membrane with detergent was developed. This method was applied to two-color flow cytometric analysis of both intraerythrocytic (IE) malaria DNA and parasite-derived antigen on the erythrocyte surface membrane. Infected erythrocytes were fixed with 0.025% glutaraldehyde followed by treatment with 1% saponin to gain access to intramembranous components and allow antibody to interact with antigen. DNA of IE parasite was subsequently stained with propidium iodide. Using this procedure cell morphology was well preserved with excellent parasite DNA staining.

Using anti-malaria antibodies which recognize ring-infected erythrocyte surface antigen (Pf155/RESA), we observed that glutaraldehyde-fixed saponin treated infected erythrocytes inhibited a variable immunofluorescence intensity as assessed by both flow cytometry and fluorescence microscopy. Ring-infected cells displayed strong immunofluorescence staining whereas a weak signal was detected on cells containing schizonts. Simultaneous measurement of parasite DNA and antigen in the infected erythrocyte membrane can facilitate the study of antigen expression in the cell membrane in association with development of IE parasites.

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## BRIEF REPORTS

# Two-Color Flow Cytometric Analysis of Intraerythrocytic Malaria Parasite DNA and Surface Membrane-Associated Antigen in Erythrocytes Infected With *Plasmodium falciparum*<sup>1</sup>

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A method for fixation of *Plasmodium falciparum* infected erythrocytes and solubilization of the erythrocyte membrane with detergent was developed. This method was applied to two-color flow cytometric analysis of both intraerythrocytic (IE) malaria DNA and parasite-derived antigen on the erythrocyte surface membrane. Infected erythrocytes were fixed with 0.025% glutaraldehyde followed by treatment with 1% saponin to gain access to intramembranous components and allow antibody to interact with antigen. DNA of IE parasite was subsequently stained with propidium iodide. Using this procedure cell morphology was well preserved with excellent parasite DNA staining.

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face antigen (Pf155/RESA), we observed that glutaraldehyde-fixed saponin treated infected erythrocytes exhibited a variable immunofluorescence intensity as assessed by both flow cytometry and fluorescence microscopy. Ring-infected cells displayed strong immunofluorescence staining, whereas a weak signal was detected on cells containing schizonts. Simultaneous measurement of parasite DNA and antigen in the infected erythrocyte membrane can facilitate the study of antigen expression in the cell membrane in association with development of IE parasites.

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**Key terms:** Fluorescein, malaria antigen, nucleic acid fluorochrome, propidium iodide, red blood cell

During intraerythrocytic (IE) development of the asexual blood stage of *Plasmodium falciparum*, there are complex cellular changes involving surface "knob" proteins (13), membrane structural components (1, 22), and expression of various surface associated parasite antigens (2, 3, 6, 10). Expression of parasite-derived antigens on infected erythrocytes is associated with both parasite survival through cytoadherence to vascular endothelium and the host response mediated by antibody and immune cells. Antibody responses to antigens on the surface of trophozoite/schizont-stage infected erythrocytes are known to be directed against strain specific or conserved determinants (epitopes) (7, 15). Identification of conserved epitopes has important

implications for malaria vaccine development and for understanding naturally acquired malaria immunity. A merozoite-derived polypeptide of 155 Kda, the ring-

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infected erythrocyte surface antigen (Pf155/RESA), is a conserved molecule (18) and is associated with the membrane of ring-stage erythrocytes. The Pf155/RESA is not accessible on the outer surface of infected erythrocytes and was first detected by microscopy using erythrocyte membrane immunofluorescence of glutaraldehyde-fixed and air-dried ring-infected erythrocytes (17). This technique was subsequently modified by using saponin or Triton X-100 to solubilize erythrocyte membrane lipid components instead of air drying (20).

Various methods have been used for detection of IE malaria parasites using nucleic acid-binding fluorochromes and flow cytometry (4, 8, 9, 14, 26). Recently we developed a simple method for fixation and permeabilization of malaria infected erythrocytes which allows simultaneous flow cytometric detection of human erythrocyte membrane antigen and IE malaria DNA (16). We report in this study a further modified immunofluorescence technique for two-color flow cytometric analysis of parasite DNA and erythrocyte surface associated parasite derived antigen.

## MATERIALS AND METHODS

### Parasite and Culture Conditions

*Plasmodium falciparum*, Thai isolate TM 178R was provided by Dr. S. Thaithong of the Department of Biology, Chulalongkorn University, Bangkok. Cultures were maintained as described previously (23) using a suspension of 5% O-positive human erythrocytes in RPMI 1640 medium (Seromed, Biochrom, Germany) supplemented with 10% heat-inactivated human serum, 2 mM L-glutamine (Flow Laboratories), 25 mM HEPES buffer (Flow Laboratories), and 20 µg/ml gentamicin at pH 7.4 in a gaseous atmosphere of 5% CO<sub>2</sub> plus 5% O<sub>2</sub> and 90% N<sub>2</sub>. In some experiments, cultures were synchronized by sorbitol treatment (11). Harvested cells were stained for flow cytometric analysis as described below. A concurrent Giemsa stained blood film was also prepared from the same culture suspension. Parasitemias were calculated from numbers of infected cells in at least 1,000 erythrocytes under light microscopy.

### Immune Serum and Monoclonal Antibody

Pooled immune serum was obtained from Thai adults living in a malaria endemic area of eastern Thailand and stored at -20°C. Non-immune serum was pooled from healthy Thai blood donors who had no history of malaria. The sera were heat inactivated and adsorbed twice with group AB+ erythrocytes at 50% cell suspension.

A malaria specific human monoclonal antibody 33G2 (IgM) recognizing Pf155/RESA as determined by erythrocyte membrane immunofluorescence and immunoblotting (24, 25) was obtained from culture supernatant of an EBV-transformed B lymphocyte clone (19).

The optimum working dilutions of immune serum and monoclonal antibody were 1:16 and 1:10, respectively. These dilutions were obtained from titration of

antibodies reacting with infected erythrocytes and determined by flow cytometry. The lowest antibody concentration at which there was maximum fluorescence was used.

### Prestaining Fixation and Detergent Treatment

Parasitized erythrocytes were washed twice with phosphate-buffered saline (PBS) and resuspended in 0.025% (v/v) glutaraldehyde (GA, Sigma Chemical Co., St. Louis, MO) in PBS at 2% hematocrit for 20 min at room temperature. The cells were then washed with PBS and resuspended in PBS containing 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) at 50% hematocrit. Half of the GA-fixed cells were resuspended in PBS containing 1% saponin (Sigma Chemical Co., St. Louis, MO) for 15 min at room temperature. These cells were designated as detergent-treated cells. Non-detergent-treated cells were processed as above but without saponin treatment. In some experiments, live infected erythrocytes were used without GA fixation.

### Immunofluorescence Staining

Ten microliters of packed cells from detergent-treated and nondetergent-treated infected erythrocytes were mixed with 40 µl of either immune serum or monoclonal antibody 33G2 and incubated at room temperature for 30 min. After washing with PBS containing 1% BSA, the cells were incubated for another 30 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (Becton Dickinson, San Jose, CA). Finally the cells were washed twice with PBS-BSA and the pellet was then resuspended in 50 µl of PBS-BSA and kept for subsequent IE parasite DNA staining.

### Intraerythrocytic (IE) Parasite DNA Staining

After fluorescence staining of parasite-derived antigen associated with the membrane of infected erythrocytes, 50 µl of stained cells were resuspended in 2 ml of PBS containing 10 µg/ml propidium iodide (PI, Molecular Probes, Inc.) and kept in the dark for at least 1 h prior to flow cytometric analysis. Specificity of the staining was checked by mounting one drop of each sample on a microscopic slide with a cover slip for UV microscopy.

### Flow Cytometric Analysis

Analysis of parasite-associated membrane antigen of infected erythrocytes and IE parasite DNA was performed using a FACScan flow cytometer (Becton Dickinson) equipped with a 15 mW argon ion laser tuned at 488 nm. In this system FITC and PI produced green and red fluorescence, respectively. Logarithmic green fluorescence was observed through a 530/30 band pass filter and simultaneously red fluorescence was detected through a 585/42 nm band pass filter. Erythrocytes were gated on the basis of their forward light scatter and granularity using logarithmic scale. Con-

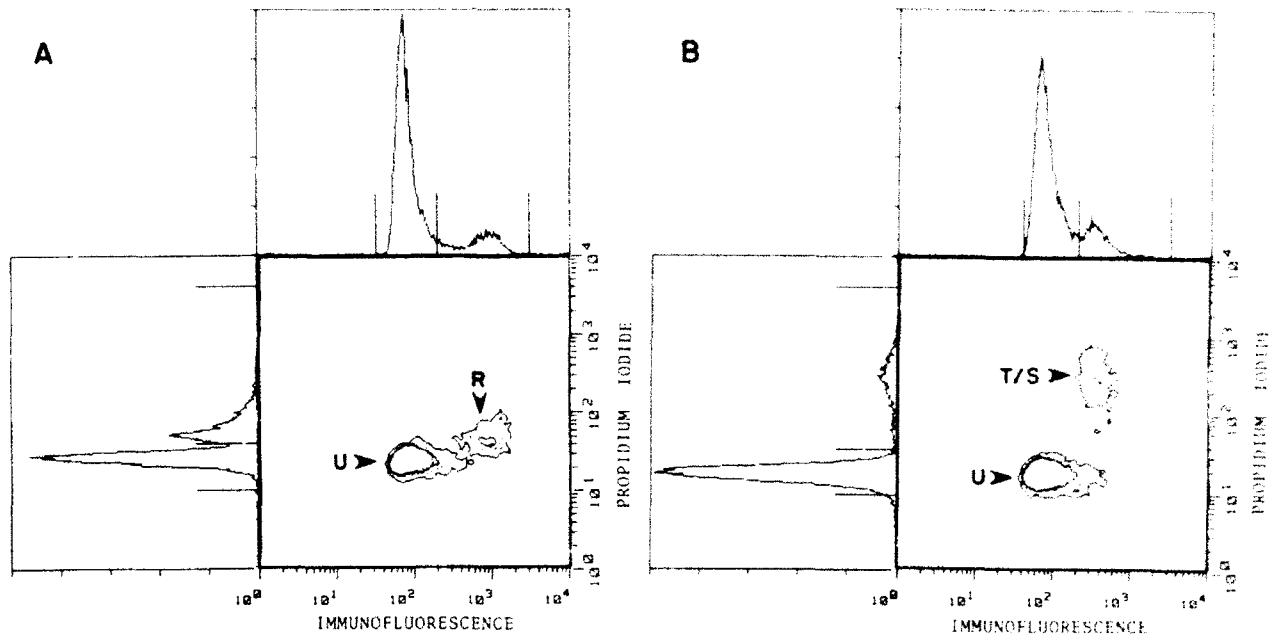


FIG. 1. Histograms and two-parameter contour plots of *Plasmodium falciparum*-infected erythrocytes from synchronous cultures stained with propidium iodide after treatment of cells with glutaraldehyde and saponin. Histograms represent cell counts against increasing intensity of immunofluorescence and propidium iodide on

the X- and Y-axis. Example is representative of seven experiments. A: Ring stage parasites, B: Schizont stage parasites. U, uninfected erythrocytes; R, ring-infected erythrocytes; T/S, trophozoite/schizont stage erythrocytes.

trol erythrocytes were prepared as above except without immune serum. To compare fluorescence intensity among samples, instrument fluorescence compensation and sensitivity were calibrated, saved, and used throughout the course of this study.

For each sample, 10,000 cells were analysed for fluorescence intensity using Consort C30 software (Becton Dickinson). Results were presented as the mean fluorescence intensity (MFI) or percent of positive cells obtained from both histogram and two-parameter cytogram analysis.

## RESULTS

### Effect of Fixation and Detergent Treatment on IE Malaria Parasite

GA fixation in combination with saponin treatment produced a characteristic increase of green and red autofluorescence in unstained erythrocytes, whereas no such changes occurred in non-fixed cells. Despite the increased fluorescent signals, no aberrations in the forward and side scatter were observed. Moreover, IE parasites could be visualized and differentiated after staining with PI. As shown in Figure 1, ring stage parasites produced a peak fluorescence with MFI of 50 (Fig. 1A). The peak fluorescence for the schizont stage (MFI of 230) occurred at a higher signal intensity than for rings (Fig. 1B). The percent parasitemias obtained by flow cytometry were significantly correlated ( $r = 0.98$ ,  $P = 0.0001$ ) with the microscopic results (Table

1). Live infected erythrocytes stained with PI showed negligible red fluorescence.

### Effect of GA and Detergent on Parasite-associated Membrane Antigens of the Infected Erythrocytes

Detergent treated and non-detergent treated erythrocytes stained with antibodies and PI were analysed by flow cytometry. In detergent treated erythrocytes, ring-stage infected cells showed higher green fluorescence than schizont-infected cells but exhibited a low red signal compared to schizont stage (Fig. 1A,B). This indicates lower DNA content in ring-stage parasites than schizont-stage parasites. It also showed that detergent-treated ring-infected erythrocytes consistently had a greater green MFI than that of erythrocytes containing schizonts. These features were also apparent in asynchronous cultures (Fig. 2). Interestingly, the percent detergent treated antigen-positive ring-infected cells was comparable to the percent parasitemia determined by PI staining (Table 2).

There was an impressive difference in antigen expression of ring-infected erythrocytes of non-detergent treated and detergent-treated cells. Both immune serum and monoclonal antibody 33G2 gave a stronger fluorescent signal on detergent-treated ring-infected cells than non-detergent treated ring-infected cells. In contrast, no distinct difference in MFI was observed between non-detergent treated and detergent-treated schizont-infected cells. No staining was observed with

Table 1  
Comparison of the Percent Parasitemia in Erythrocyte Cultures of *Plasmodium falciparum* as Determined by Microscopy and Flow Cytometry

Culture number	% parasitemia	
	Microscopy <sup>a</sup>	Flow cytometry <sup>b</sup>
1	3.1	4.3
2	3.6	2.7
3	4.5	5.6
4	5.8	7.0
5	7.5	8.9
6	8.3	9.8
7	12.2	14.5
8	15.4	12.2
9	17.7	19.5
10	20.1	22.6
11	22.7	22.5
12	29.3	28.4

<sup>a</sup>Parasites stained with Giemsa.

<sup>b</sup>Parasites stained with propidium iodide.

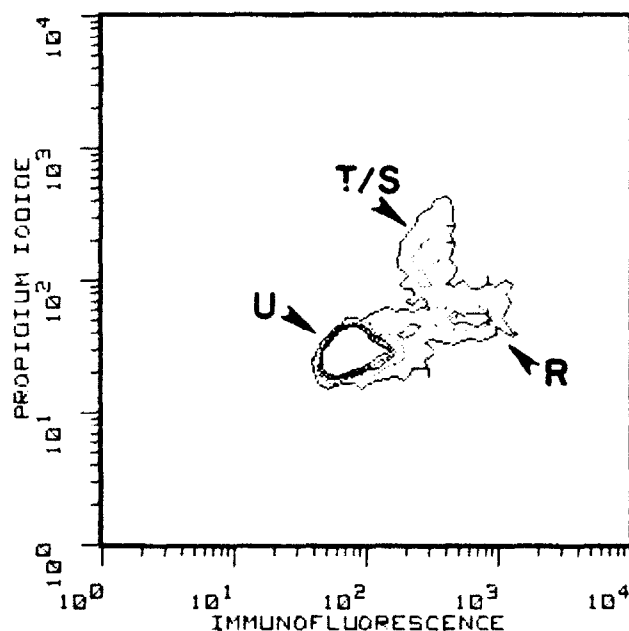


FIG. 2. Representative two-parameter contour plot of asynchronous glutaraldehyde/saponin treated *Plasmodium falciparum*-infected erythrocytes following indirect staining of cells with immune serum (X-axis) and propidium iodide (Y-axis). U, uninfected erythrocytes; R, ring-stage erythrocytes; and T/S, trophozoite/schizont stage erythrocytes.

non-infected erythrocytes. Non-detergent treated ring-infected erythrocytes showed a weak signal. Infected erythrocytes of both ring and schizont stages reacted with non-immune serum were negative for green fluorescence consistent with the above findings but did produce a red signal for parasite DNA.

Microscopic examination confirmed that only ring-infected erythrocytes were stained with antibody. Membranes of detergent treated ring-infected erythro-

Table 2  
Comparison of Percent Positive Ring-Infected Erythrocytes Bearing Parasite-Derived Surface Antigen and Percent Parasitemia Assessed by Flow Cytometry Using Propidium Iodide Staining

Culture number	% positive infected cells bearing parasite antigen	% parasitemia
1	15.7	15.0
2	13.2	12.9
3	21.3	20.7
4	18.1	17.5
5	7.9	7.2
6	11.5	10.9
7	15.4	14.8

cytes showed a strong rim-like fluorescence, whereas detergent-treated erythrocytes containing schizonts showed only weak fluorescence (Fig. 3).

## DISCUSSION

To better understand the relationship between expression of new antigenic determinants in the membrane of *P. falciparum*-infected erythrocytes and the IE development of the parasite, we used a novel fixation and detergent (17, 20) technique with two-color staining for erythrocyte membrane antigen and IE parasite DNA (16). We first examined the effects of GA fixation and saponin treatment on the detection of IE malaria DNA and parasite-derived antigen in infected erythrocytes. Saponin has been shown to produce defects in the cell membrane rendering it permeable and causing cell damage characterized by a decrease of forward and right-angle light scatter (19, 21). This was not observed in erythrocytes fixed with GA prior to saponin treatment. Primary fixation of cells with GA thus preserved cell integrity. However, fixing cells with GA for later immunofluorescence staining and detergent treatment caused a high auto-fluorescent background. Aldehyde fixatives are known to react with a variety of amines found in erythrocytes to produce non-specific fluorescent material (12). Despite this effect, the DNA signal obtained with PI staining was acceptable. Percent parasitemia determined by flow cytometry was comparable to microscopy of Giemsa blood smears (Table 1).

Staining of parasite-derived surface antigen on erythrocytes recognized by immune serum was found to be different following detergent compared to non-detergent treatment. Antiserum gave strong fluorescence on GA-fixed cells that had been saponin treated. Ring-infected erythrocytes showed strong fluorescence, whereas schizont-infected erythrocytes were weakly stained (Fig. 1). The presence of RESA was confirmed by IFA (Fig. 3), in which the localization of parasite-derived antigen reacting with immune serum or monoclonal antibody 3G2 was found to be mainly associated with the ring-infected erythrocytes. RESA did not appear to be expressed on the surface of intact, non-fixed infected erythrocytes. The detection of RESA on detergent-treated ring-infected erythrocyte membrane

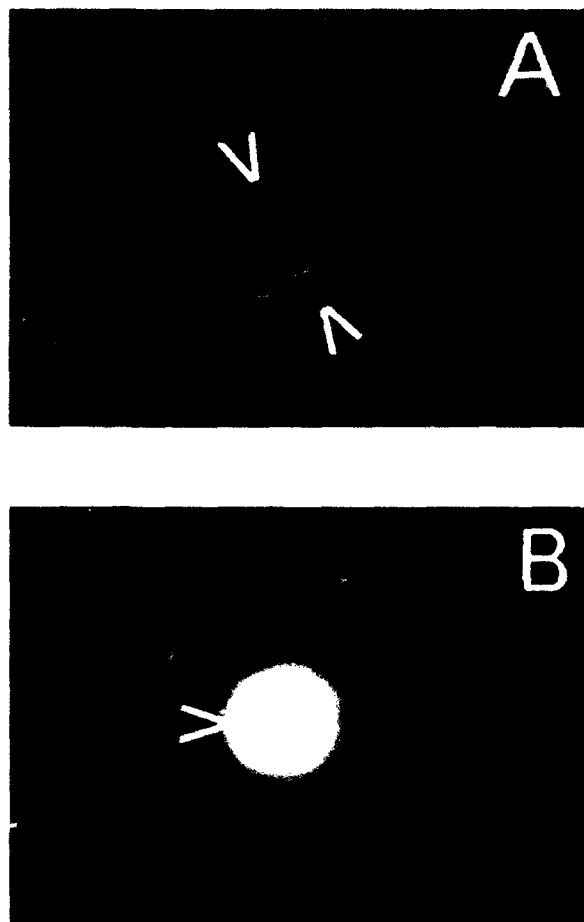


FIG. 3. Indirect immunofluorescence microscopy of glutaraldehyde-fixed and saponin-treated *Plasmodium falciparum*-infected erythrocytes reacted with immune serum. The parasite nuclei were counterstained with propidium iodide (Arrows). A shows both surface immunofluorescence of ring-infected erythrocytes and intracellular fluorescence of parasite nuclei, whereas B shows only intracellular nuclei staining of schizont-infected erythrocytes.

by flow cytometry confirmed earlier studies indicating the Pf155/RESA was not exposed on the surface of infected erythrocytes (5, 17, 20) but that the C-terminal repeated region of this antigen was associated with the membrane cytoskeleton below the outer layer of the plasmalemma.

In addition to the effect of saponin on expression of parasite-derived antigen, the method also gave a positive correlation between percent antigen positive ring-infected erythrocytes and percent parasitemia (Table 2). Schizont-containing erythrocytes and non-detergent treated ring-infected cells showed no correlation as schizonts exhibited a very weak immunofluorescence signal. This method could thus be used to examine the antigenic complexity of the surface determinants exposed on infected erythrocytes reactive with immune sera or monoclonal antibodies. Moreover, the ability of antibody to react with the antigen located below the

outer surface membrane of erythrocytes shown by this method may be applied to study other membrane integrated proteins of erythrocytes either in malaria infection or other diseases involving erythrocytes.

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